



Ottawa / Hull / K1A 0H9

(21)	(A1)	2,108,705
(22)		1993/10/19
(43)		1994/05/07

(51) INTL.CL. ⁵ G01N-033/557; G01N-033/553; C12Q-001/68; G01N-021/17;
G01N-021/59; C07K-017/14

(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Biologically Recognizing Layers on New TiO₂ Waveguide
for Biosensors

(72) Barner, Richard - Switzerland ;
Fattinger, Christof - Switzerland ;
Huber, Walter - Switzerland ;
Hübscher, Josef - Switzerland ;
Schlatter, Daniel - Switzerland ;

(71) F.Hoffmann-La Roche AG - Switzerland ;

(30) (CH) 3444/92 1992/11/06

(57) 11 Claims

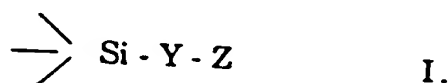
Notice: This application is as filed and may therefore contain an
incomplete specification.



2108705

Abstract

The present invention is concerned with the coating of dielectric TiO_2 wave guides with biologically recognizing elements to give biosensors having high sensitivity and specificity for an analyte molecule. The coating consists of an organic carrier layer to which receptor molecules are bonded, the carrier layer having an ordered monomolecular layer which consists of molecules of general formula I



This layer is bonded directly to the TiO_2 wave guide via the Si atom or, if desired, is bonded to a TiO_2 wave guide via an intermediate layer. The receptor molecules are biological molecules having recognizing properties, such as antigens, antibodies, receptors, DsDNA, ssDNA. The arrangement of the receptor molecules on the sensor surface can be not only two dimensional but also three dimensional, and the receptor molecules can be immobilized non-directed or directed on the organic carrier layer.

The present invention is concerned with optical biosensors and with a method for their production; in particular, it is concerned with methods and compounds for applying biologically recognizing elements to TiO_2 wave guides which are used for these novel optical sensors.

According to definition, a biosensor is a device consisting of a transducer and a biologically recognizing element (Trends in Biotechnol. 2 (1984), 59). Such biosensors can be used to determine analyte concentrations e.g. in human and veterinary diagnostics, in environmental analysis and in the analysis of food or in biochemical research for the quantification of intermolecular interactions of biologically active substances (e.g. antibody-antigen interaction, receptor-ligand interaction, DNA-protein interaction etc.).

The function of the biologically recognizing element of a biosensor is to recognize an analyte molecule in solution and to bind to it (so-called affinity sensor) or to catalytically modify it (so-called enzymatic, metabolic sensor). The change in the biologically recognizing element which accompanies this is recognized by the transducer (signal transformer), which is in close contact with the element, and is converted into a processable signal.

One class of such transducers detects alterations in the optical properties of the biologically recognizing element (e.g. absorption, refraction) by means of an optical surface wave, which is conducted in a wave-guiding layer along the interface of the transducer/recognizing element. This class of transducers can be divided into two groups on the basis of different wave-guiding layer structures. A first group embraces transducers in which this wave-guiding structure is the interface between a metal and a dielectric. The wave which is conducted at this interface is the surface plasmon (Sensor and Actuators 4 (1983) 299). The second group embraces transducers having dielectric

wave-guiding layers. The optical waves which are conducted are wave guide modes (Opt. Lett. 9 (1984) 137; Sensors and Actuators A, 25 (1990) 185; Sensors and Actuators B, 6 (1992) 122; Proc. Biosensors 92, extended abstracts, pp 339 & pp 347).

The present invention is concerned with biosensors which are based on dielectric wave guides. The basic principle of such transducers can be explained on the basis of the field distribution of the modes which are conducted in such wave guides. The electric field of the conducted mode is not limited solely to the geometric dimensions of the wave guide, but has so-called evanescent components, i.e. the field distribution of the conducted modes dies away exponentially in the media adjacent to the wave guide (e.g. in the substrate or in the superstrate in which the wave-guiding layer is situated). Changes in the optical properties of the substrate or superstrate adjacent to the wave guide within the transmission range of the evanescent field influence the propagation of these modes and can be detected by suitable measuring instruments. When the superstrate contains the biologically recognizing element within the transmission range of the evanescent field, then the changes in the optical properties of this element which accompany the binding or modification can be detected by this optical, surface-sensitive method and calibrated in terms of an analyte concentration.

It is known from the literature that the higher are the surface specificity and the surface sensitivity of the method, then the smaller is the effective density of the wave-guiding layer (monomode wave guide) and the higher is the jump in the refractive index at the interface of the wave guide/substrate and wave guide/superstrate. Having regard to its high refractive index, TiO_2 is therefore especially suitable as a material for such wave guides and it has been shown recently (Proc. Materials res. Soc. Spring Meeting, San Francisco, 1992) that by using PICVD technology wave-guiding films can be produced from this material with a refractive index of 2.45 for sensorics.

The present invention is concerned with the coating of such TiO_2 wave guides with biologically recognizing elements, there being obtained biosensors of high sensitivity and specificity for an analyte molecule. The basis for these recognizing elements is that a so-called recognizing molecule (e.g. antibody, membrane receptor, ssDNA sample etc) comes into play for a selective recognition and binding (and/or modification) of an analyte molecule (e.g. antigen, ligand, active substance, ssDNA, etc). These recognizing molecules can be used not only in their naturally occurring and isolatable form, but also in a chemically or biotechnologically prepared form.

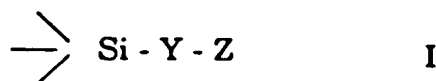
The object of the present invention is to provide an optical biosensor and a method for its production, the biosensor consisting of a TiO_2 wave guide and an organic carrier layer with the receptor molecules bonded thereon and this carrier layer satisfying the requirements of optical biosensors, i.e.

- its layer thickness is not greater than the transmission range of the evanescent field of the mode conducted into the wave guide;
- its construction shows optical homogeneity with respect to the light conducted into the wave guide;
- it shows chemical resistance to media with which it comes into contact (sera, fermentation solution, etc);
- the recognizing molecules are anchored to it such that their natural activity is maintained;
- the recognizing molecules are anchored to it such that they are not lost by dissociation in contact with the sample.

The object of the invention is to provide corresponding recognizing elements on the novel TiO_2 wave guides appropriate

to the different analyte molecules and, respectively, recognizing receptor molecules.

In accordance with the invention the object is achieved by providing an optical biosensor consisting of a dielectric wave guide and an organic carrier layer to which receptor molecules are bonded, wherein the organic carrier layer and the receptor molecules bonded thereon form an ordered monomolecular layer and the carrier layer consists of molecules of general formula I



and wherein the molecules of the carrier layer are bonded to a TiO_2 wave guide directly via the Si atom or, if desired, are bonded to a TiO_2 wave guide via an intermediate layer.

Examples of optical biosensors and a process for the production of the biosensors in accordance with the invention will be described hereinafter.

For the construction of the organic carrier layers having the required properties, the TiO_2 wave guide surface is firstly provided with a homogeneous organic supplementary layer. The compounds used for the derivatization of the TiO_2 surface are silanes of general formula II



wherein

$-\text{Si}(\text{R}^1\text{R}^2\text{R}^3)$ represents a coupling group to the TiO_2 layer and R^1 , R^2 , R^3 can be alkyl, alkoxy or halogen, but at least one of these residues is either alkoxy or halogen,

$-\text{Y}$ is a spacer group and as such represents either an alkylene chain $-\text{CH}_2-(\text{CH}_2)_n-\text{CH}_2-$, a fluoroalkylene chain $-\text{CH}_2-(\text{CF}_2)_n-\text{CH}_2-$ or $-\text{CH}_2-(\text{CF}_2)_n-\text{CF}_2-$ with $n = 1-30$, an oligoethylene chain $-[(\text{CH}_2)_{n'}-\text{O}-(\text{CH}_2)_{n''}]_m-$ with n' , $n'' = 2-6$ and

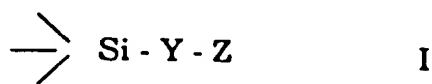
m = 2-6 or a combination of alkylene, fluoroalkylene or oligoalkylene glycol and

-X is either hydrogen or fluorine or a chemically reactive group which is compatible with $-\text{Si}(\text{R}^1\text{R}^2\text{R}^3)$ such as e.g. carboxylic acid halide ($-\text{COHal}$), olefin ($-\text{CH}=\text{CH}_2$), nitrile ($-\text{CN}$), thiocyanate ($-\text{SCN}$) and thioacetate ($-\text{SCOCH}_3$) or, when $\text{R}^1, \text{R}^2, \text{R}^3 =$ alkoxy, also amine ($-\text{NH}_2$).

After the addition of the compounds to the TiO_2 surface, these groups can also be converted by suitable subsequent chemical treatments into groups which are not compatible with $-\text{Si}(\text{R}^1\text{R}^2\text{R}^3)$ such as e.g. into azide and further into amine, or nitrile into amine, or halogen into thiocyanate and further into thiol, or thioacetate into thiol or olefin into epoxide, diol, halide, dihalide or carboxylic acid etc.

Other molecules can be coupled to the original group X or to the group X subsequently treated as just described to give an organic carrier layer to which the receptor molecules are bonded.

An optical biosensor is obtained in which the organic carrier layer forms ordered monomolecular layers and consists of molecules of general formula I.



In this formula Z signifies the groups:

- hydroxyl, carboxyl, amine, methyl, alkyl, fluoroalkyl groups;
- derivatives of hydrophilic short-chain molecules such as oligovinyl alcohols, oligoacrylic acids, oligoethylene glycols;
- derivative of mono- or oligo-saccharides with 1-7 sugar units;

- carboxyglycoside derivatives;
- aminoglycoside derivatives such as fradiomycin, kanamycin, streptomycin, xylostatin, butirosin, chitosan
- derivative of hydrogel-forming groups of natural or synthetic origin such as dextran, agarose, alginic acid, starch, cellulose and derivatives of such polysaccharides or hydrophilic polymers such as polyvinyl alcohols, polyacrylic acid, polyethylene glycols and derivatives of such polymers.

It has surprisingly been found that the compounds of formula II are outstandingly suitable for applying to TiO_2 monomolecular, densely packed, ordered organic films having the quality required for optical sensorics.

For low-molecular representatives of the compounds of formula II this coating is preferably carried out from the gas phase (chemical vapor deposition (CVD) method). For high-molecular compounds this coating can also be carried out from the liquid phase. In order, however, in the coating of the TiO_2 wave guide to achieve a homogeneity which is sufficient for use in optics, the solvent used must be coordinated with the compounds of formula II.

The biologically recognizing elements in biosensorics are generally constructed from an organic carrier layer which is covalently linked with the substrate (transducer surface) and to which biologically recognizing molecules are absorbatively or, preferably, covalently bonded. As will be evident from the following, in biosensorics the specific construction of a biologically recognizing element is on the one hand closely linked with the type of analyte molecule to be detected and thus with the nature of the receptor molecule which is used for the detection and is on the other hand, however, also dependent on the

problem position to be resolved with a particular biosensor for a receptor/analyte molecule pair.

The biologically recognizing elements, which are claimed here for use in optical biosensorics in combination with TiO_2 wave guides, are divided into two main classes A and B, with each of these main classes being divided into two sub-classes A1, A2 and B1, B2. The criterium relevant for the assignment of a recognition element to one of the main classes relates to the arrangement of the receptor molecules on the sensor surface. In the first class (A) the receptor molecules are ordered approximately in one plane (two dimensional arrangement) on the surface of the optical transducer. Such a two dimensional arrangement of the receptor molecules results only when the dimension of the organic carrier layer perpendicular to the transducer surface is not substantially greater than the molecular size of the receptor molecule bonded to this carrier layer. In recognizing elements of class B the immobilized receptors have a three dimensional arrangement. This three dimensional arrangement can only be realized with a carrier layer having a thickness which is substantially greater than the molecular dimensions of the receptor molecule and which proves to be permeable for the receptor molecule. This type of carrier layer can be denoted as a porous, three dimensional matrix. The criterion for the assignment to one of the sub-classes (A1, B1 or, respectively, A2, B2) relates to the manner in which the receptor molecules are immobilized on the carrier layer; the differentiation being a non-directed (A1, B1) and a directed (A2, B2) mode of immobilization, the term immobilization being used not only for an absorptive but also for a covalent bonding to the organic carrier layer. Non-directed immobilization of a receptor molecule to the organic carrier layer signifies that in the bonding of the receptor molecule to the organic carrier layer no regard is had to particular structural features of the receptor molecule, i.e. the immobilization takes place at any position on the surface of the receptor molecule. Directed immobilization signifies that in the immobilization of the receptor molecule regard is had to the analyte-recognizing domains and for the immobilization those

structural elements are chosen which are well separated spatially from the analyte-recognizing domains.

As mentioned earlier, each of these different types of biologically recognizing elements has its specific suitability for use in different fields or investigational areas of bioanalytics. This will be substantiated using some examples:

A three dimensional matrix permits the immobilization of a larger number of receptor molecules per surface unit. Since the total number of receptor molecules per surface unit in the case of directed biosensors determines the steepness of the sensor curve and consequently the analytical capability in the concentration range relevant to the sensor, a sensor which is equipped with a three dimensional recognizing element is accordingly preferred for an exact determination of an analyte concentration (e.g. in diagnostic use). A three dimensional matrix can, however, also be of disadvantage when the analyte molecule to be detected possesses several repetitive epitopes. The binding of this analyte molecule to several receptor molecules in the outer regions of the element then leads to a cross-linking of the carrier layer, whereby the access to free binding sites on the inside of the recognizing element is impeded for subsequent analyte molecules. The disadvantage of a three dimensional arrangement of receptor molecules is also obvious in a quantitative representation of the kinetics of the binding process between a receptor molecule and an analyte molecule. The time-dependent sensor response observed in such an investigation using a three dimensional matrix can also be marked by the hindered diffusion of the analyte molecule in this matrix.

In an analogous manner, advantages and disadvantages of a directed or non-directed immobilization can be demonstrated in different bioanalytical investigations. For the detection of an antigen in immunodiagnosics it is without doubt important to immobilize the antibody used for the detection on the surface such that the antigen-recognizing domains are not influenced by the immobilization, e.g. over the Fc part which is well separated

from the antigen-recognizing domains. When, however, a biosensor is used to test for the presence of an ensemble of polyclonal antibodies against one and the same antigen, then it is convenient to immobilize the antigen in a non-directed manner, since thereby all epitopes of the antigen are equally available for recognition by the antibody in solution.

As a further embodiment of the invention it ensues accordingly that the TiO_2 surfaces for the construction of the biologically recognizing elements of type A1, A2, B1 and B2 must be provided with organic carrier layers which permit the directed or non-directed immobilization of receptor molecules in a two or three dimensional arrangement.

In addition to the aforementioned requirements relating to the dimensions and permeability of a carrier layer, which is provided for the two or three dimensional arrangement of receptor molecules, these organic carrier layers for the immobilization of the receptor molecules must also satisfy chemical and, respectively, physicochemical requirements and must have

a) reactive groups by means of which receptor molecules can be covalently anchored to/in the two/three dimensional carrier layer

and

b) functional groups or molecules and/or molecular associations which permit an efficient concentration of receptor molecules to/in this two/three dimensional matrix before the covalent anchoring, so that the immobilization of receptor molecules can be carried out from dilute solutions.

ad a): A large number of reactive groups which can be used for such a covalent immobilization are known from the literature. A differentiation is made between groups which are per se chemically active and which can enter into a bonding with

functional groups on the receptor molecule, such as e.g. amino, hydrazine or hydrazide groups on the carrier layer, which can react with aldehyde groups on the receptor molecule, and vice versa, activated disulphide bonds on the carrier layer which react with free thiol groups on the receptor molecule, carboxylic acid halides or activated carboxylic acid esters on the carrier layer which react with amino groups on the surface of the receptor molecule etc, and groups which react with functional groups on the receptor molecule after an in situ activation (chemical or photochemical activation), such as e.g. aziridine or phenylazide which are converted by a photochemical activation into reactive carbene or nitrene.

ad b) such a concentrating property can be conferred to a carrier layer in various ways, e.g. by ionic groups by means of which receptor molecules of opposite total charge are concentrated on the basis of a Coulomb interaction with the ionic groups of the carrier layer in an approximately non-directed manner,

or by molecular associations which confer a hydrophobic character to a carrier layer such that receptor molecules having hydrophobic domains are concentrated via these domains at these surfaces in a directed manner,

or by metal complexes having non-saturated coordination spheres which are saturated by particular functional groups or domains of a receptor molecule and thereby a directed concentration on the carrier layer is effected,

or by molecules having the capacity of a molecular recognition (e.g. protein A, protein G, streptavidin, antibodies against particular epitopes of a recognition molecule etc), which have a high affinity to particular domains of a recognition molecule and which concentrate a receptor molecule as a result of this affinity to/in the carrier layer in a directed manner.

It has surprisingly been found that in the coating in accordance with the invention of planar TiO_2 wave guides there are obtained organic layers which have an analogous construction and a comparable arrangement to organic monolayers which are applied using the compounds of formula II to materials such as silicone, silicone oxide and aluminium oxide (Advanced Materials 2 (1990) 573; Langmuir 8 (1992) 947) or to organic monolayers which can be applied using functionalized thioalkanes to gold surfaces (Langmuir 6 (1990) 87). This class of organic layers is denoted by the term "self assembled monolayer" in the technical literature. Under the described conditions there are obtained organic monolayers on the TiO_2 surfaces in which the compounds of formula II are covalently bonded with the TiO_2 via the terminal Si atom and the spacer group Y having the reactive group X stands clear of the surface. The compounds of formula II bond to the TiO_2 layers via the reactive group $(\text{R}^1\text{R}^2\text{R}^3)\text{-Si-}$ in that at least one of the groups $(\text{R}^1, \text{R}^2, \text{R}^3)$ reacts with free hydroxyl groups on the surfaces. In order to obtain a dense packing and resistant layers, it is accordingly important to pre-treat the TiO_2 surface in a suitable manner so that a high density of hydroxyl functions results on the surface. The layers obtained on the TiO_2 surfaces with the compounds of formula II have been found to be stable in organic solvents and in aqueous media with a $9 > \text{pH} > 1$. In basic aqueous media $\text{pH} > 10$ their stability decreases, namely with decreasing number of reactive groups R at the terminal Si atom of the compounds of formula II.

With reference to their adhesion to the TiO_2 surface, these monolayers are also stable towards reduction agents such as BH_3 or LiAlH_4 and, respectively, towards oxidation agents such as aqueous permanganate solution or perchlorate solution.

These monolayers of organic compounds covalently bonded with the surface of the TiO_2 can be used directly as two dimensional carrier layers when the reactive groups are those which react with functional groups on receptor molecules (e.g. acid halide, epoxide, aldehyde, hydrazide). Otherwise, these groups must be modified in a suitable manner (e.g. olefin into

carboxylic acid, halide, epoxide, or halide into azide and further into amine, or thiocyanate into thiol) and/or activated (e.g. carboxylic acid into activated ester). Such procedures for the modification or activation of functional groups on surfaces are known from the literature (e.g. IEEE Transactions on Biomedical Engineering 35 (1988), 466; Analytica Chimica Acta 229 (1990) 169; Analytica Chimica Acta 228 (1990) 107; Biosensors and Bioelectronics 7 (1991) 207, Langmuir 6 (1990), 1621). A further possibility of modification comprises adding heterobifunctional photoreagents (e.g. compounds having phenylazido or aziridino groups as photoreactive groups and an activated carboxylic acid as chemically reactive groups) via the chemically reactive groups on the functional groups X to give a surface to which, during exposure to light, receptor molecules can be immobilized on the surface (Journal of Photochemistry and Photobiology, B: Biology 7 (1990) 277).

A variant of the monofunctional carrier layer described above, which leads to recognizing elements of type A, comprises producing a surface having different functional groups using compounds of formula II so that a multifunctional organic monolayer is obtained. Such a mixed layer can be produced by using a mixture of compounds of formula II for the coating of the TiO_2 surface or by a subsequent chemical modification in which the chemically reactive groups X on the surface are only transformed partially into a group X'. Such a mixed layer can then carry chemically reactive (such as e.g. activated carboxylic acid) or activatable (such as e.g. aziridine or phenylazide) groups and at the same time also functional groups, molecules and/or molecular associations which permit the aforementioned concentration of receptor molecules for the immobilization.

For example, an organic monolayer which carries hydroxyl groups as functional groups X can be produced in a first coating step. This is carried out in a simple manner by treating the TiO_2 surface with a compound of formula II which carries a double bond as a functional group X. This double bond is converted into a diol group by treating this surface with peracids (e.g. chloroper-

benzoic acid and subsequent treatment with acidic-aqueous solutions of pH = 3). Upon treatment of this surface with compounds of formula II in which the spacer group is a perfluoro-alkane chain and the functional group is a fluorine atom there results a surface which bonds receptor molecules preferably over hydrophobic domains. The thereby resulting recognizing element has the characteristic properties of an element of type A2 (directed immobilization of receptor molecules in a two dimensional arrangement). It has e.g. surprisingly been found that membrane proteins concentrated and anchored on such surfaces preferably bond to this surface via the transmembrane part and thus preserve approximately 100% of their natural activity.

An alternative modification of such organic monolayers comprises the addition of biomolecules which recognize and bond specific, non-analyte binding domains of the receptor molecules to be immobilized. A monolayer of protein A can be immobilized e.g. on an organic monolayer via activated carboxylic acid groups for the concentration of antibodies under suitable buffer conditions the antibodies are adsorbed via their F_c part on protein A. By unspecific coabsorption of molecules, which carry photo-activatable groups (e.g. BSA modified with phenylazido compounds), these adsorbed antibodies can subsequently be anchored to the surface in a light-induced reaction and there again results a recognizing element of type A2.

In a preferred modification of such bifunctional carrier layers, which leads to recognizing elements of type A1, the reactive groups of the organic monolayer are used to anchor low-molecular ($MW > 1500$) hydrophilic molecules to this surface. Preferred representatives of these short-chain, hydrophilic compounds are low-molecular polymers such as oligovinyl alcohols, oligoacrylic acids and oligoacrylic acid derivatives, oligoethylene glycols and low-molecular natural compounds such as monosaccharides, oligosaccharides having 2-7 sugar units, or carboxyglycosides or aminoglycosides (such as e.g. fradiomycin, kanamycin, streptomycin, xylostasin, butirosin, chitosan etc). By an addition of such low-molecular compounds there are obtained

carrier layers which still retain their two dimensional character, but simultaneously have a high degree of biocompatibility. It has surprisingly been found that such low-molecular, hydrophilic compounds are outstandingly suitable for the construction of two dimensional carrier layers to which receptor molecules can be concentrated and anchored from dilute solutions when these compounds are equipped with ionic groups (e.g. carboxylate) and with reactive groups X' (e.g. chemically reactive groups such as aldehyde, epoxide, activated ester etc or photochemically reactive groups such as aziridine or phenylazide). In this manner there is then obtained e.g. a recognizing element of type A1.

A suitable modification of such hydrophilic surfaces occurs especially readily when the aforementioned aminoglycosides are used. These aminoglycosides, most of which have antibiotic activity, are generally synthesized from 1-5 sugar units which are derivatized with one or more amino groups. One of these amino groups can be used to immobilize the aminoglycoside on the organic monolayer described above when this monolayer carries suitable reactive groups (e.g. carboxylic acid halide, activated carboxylic acid, aldehyde). The remaining amino groups can be modified in such a manner that the carrier layer subsequently has the mentioned bifunctionality (concentration, bonding). In a simple procedure, succinic anhydride can be added e.g. to the amino groups. Some of the thereby resulting free carboxylic acid functions can be modified for the chemical bonding by conversion into the N-hydroxysuccinimide derivative (alternatively, photochemically active groups can also be added), while the non-modified free carboxylic acid functions can be used in the form of a carboxylate in order to concentrate receptor molecules having a positive total charge on the carrier layer. It has surprisingly been found that the receptor molecules (recognizing element of type A1) immobilized on such a carrier layer in a non-directed manner generally exhibit a very much higher binding activity for the analyte molecule than receptor molecules which are immobilized directly on a carrier layer prepared with a compound of formula II.

Low-molecular, hydrophilic compounds such as mono- and oligosaccharides, which in their native form carry neither reactive groups for the directed immobilization nor functional groups, molecules or molecular associations for the concentration, can be modified in a suitable manner, with such a modification being generally effected more efficiently when it is not carried out on the compound already anchored to the surface.

A typical procedure will be demonstrated using dextran 1500 (7 glucose sub-units) by way of example. This dextran can be activated in solution (e.g. DMSO) by converting the hydroxyl groups into hydroxylate groups effective for a methylcarboxylation. Since this activation takes place using NaH in a very basic medium, it can not be carried out directly on the solid phase having regard to the aforementioned instability of the organic monolayers on TiO_2 . The externally methylcarboxylated dextran 1500 can, however, be anchored very simply to an organic monolayer which carries e.g. epoxide groups, there being obtained two dimensional carrier layers having a high concentration of carboxyl groups which can be used in an analogous manner for the construction of recognizing elements of type A1 such as the organic monolayers modified with aminoglycosides and succinic acid.

Such carrier layers constructed using these low-molecular, hydrophilic compounds can also be modified further to carrier layers in order to achieve a concentration in a directed manner (recognizing elements of type A2).

For example, the carboxyl groups introduced via aminoglycosides or oligosaccharides can be used to anchor biomolecules (e.g. protein A, protein G, streptavidine etc) to the surfaces, which have a high affinity to one domain of the receptor molecule which is to be immobilized subsequently.

In addition to use as two dimensional carrier layers for receptor molecules, these aforementioned organic layers are also

suitable as a basis for the construction of three dimensional carrier layers which lead to the recognizing elements of type B.

The conversion of the two dimensional carrier layer into a three dimensional carrier layer is carried out by the addition of long-chain synthetic or natural hydrophilic polymers which are capable of forming a porous, three dimensional matrix in the nature of a hydrogel of the surface of the transducer. Typical representatives of suitable natural polymers are e.g. polysaccharides such as dextran, agarose, alginic acid, starch, cellulose or derivatives of such polysaccharides such as methylcarboxylated derivatives or synthetic, hydrophilic polymers such as polyvinyl alcohol, polyacrylic acid, polyethylene glycol.

It is important that these long-chain polymers such as the low-molecular, hydrophilic compounds are also provided with reactive groups X which permit an anchoring of receptor molecules to these three dimensional carrier layers. In a preferred embodiment this carrier layer carries, moreover, ionic groups or directing molecules and/or molecular associations which permit the concentration of receptor molecules in a non-directed (type B1) or directed (type B2) manner.

For example, dextran 500000 can be methylcarboxylated and subsequently anchored to an organic monolayer which is modified with epoxide groups. There is thus obtained an about 100 nm thick, porous carrier layer having carboxylic acid groups of which some, as carboxylate, permit the concentration of biomolecules having a positive total charge and the remainder, in activated form, can be used for the subsequent covalent anchoring.

If desired, the biologically recognizing element in accordance with the invention can be applied to the optical TiO_2 wave guide via a thin intermediate layer ($d < 20 \text{ nm}$) of SiO_2 or Al_2O_3 .

The following Examples illustrate the invention in more detail:

1. Application of densely packed, organic monolayers to a TiO_2 wave guide.

1.1. Formation of an organic monolayer on TiO_2 surfaces by treatment with $\text{Cl}(\text{CH}_3)_2\text{Si}-(\text{CH}_2)_{11}-\text{COCl}$ in a CVD process:

For the application of the compounds of formula II from the gas phase, a reaction vessel is prepared which can operate at a pressure of 10-5 mbar and in which the sample to be coated can be brought to temperatures between 30-100°C. This reaction vessel is attached to an evacuable, heatable supply vessel in which the compound used for the coating can be placed (if desired, the apparatus can also be equipped with several such supply vessels).

For the coating, the substrate is introduced into the reaction vessel. After introducing the silane $\text{Cl}(\text{CH}_3)_2\text{Si}-(\text{CH}_2)_{11}-\text{COCl}$ into the supply vessel, the supply vessel and reaction chamber are brought to an operating pressure of 10-5 mbar. The sample to be coated is heated to 100°C. After heating the reagent in the supply vessel to 50°C, the surface is treated for 1 h. with reagent from the gas phase. Subsequently, the reagent flow is stopped and the sample is treated in a vacuum at 150°C for 15 min.

(The detection of an organic monolayer on the surface is carried out by XPS and contact angle measurements).

1.2. Formation of an organic monolayer on TiO_2 surfaces by treatment with $\text{Cl}(\text{CH}_3)_2\text{Si}-(\text{CH}_2)_6-\text{CH}=\text{CH}_2$ in a CVD process:

The procedure described under 1.1. is used for the coating of the surface.

1.3. Formation of an organic monolayer on TiO_2 surfaces by treatment with $(\text{CH}_3\text{O})_3\text{Si}-(\text{CH}_2)_3-\text{NH}_2$ in a CVD process:

The coating takes place using the corresponding compound according to the procedure described under 1.1.

1.4. Formation of an organic monolayer on TiO_2 by treatment with a solution of $\text{Cl}(\text{CH}_3)_2\text{Si}-(\text{CH}_2)_{11}-\text{COCl}$.

A 0.5% (v/v) solution of $\text{Cl}(\text{CH}_3)_2\text{Si}-(\text{CH}_2)_{11}-\text{COCl}$ in CCl_4 is placed in a reaction vessel under an inert gas atmosphere. The surface to be coated is brought into contact with this solution for 25 min. under an inert gas. After this treatment, the surface is washed with CCl_4 , ethanol and water.

1.5. Formation of an organic monolayer on TiO_2 by treatment with a solution of $\text{Cl}_3\text{Si}-(\text{CH}_2)_6-\text{CH}=\text{CH}_2$:

A 0.5% solution (v/v) of $\text{Cl}_3\text{Si}-(\text{CH}_2)_6-\text{CH}=\text{CH}_2$ in hexadecane is prepared in a reaction vessel under an inert gas atmosphere. The surface to be coated is brought into contact with this solution for 5 min. under an inert gas. After this treatment, the surface is washed with hexadecane, hexane and ethanol.

1.6. Formation of an organic monolayer on TiO_2 by treatment with a solution of $\text{Cl}(\text{CH}_3)_2\text{Si}-(\text{CH}_2)_7-(\text{CH}_2-\text{O}-\text{CH}_2)_2-\text{CH}_2-\text{O}-\text{CH}_3$:

The coating is carried out according to the procedure described under 1.4.

1.7. Formation of an organic monolayer on TiO_2 by treating the surface with a solution of $\text{Cl}_3\text{Si}-(\text{CH}_2)_8\text{Br}$:

The coating is carried out according to the procedure described under 1.4.

2. Modification of functional groups on organic monolayers on TiO_2 prepared according to the procedure described under 1.

2.1. Conversion of olefins into epoxides:

A TiO_2 surface treated according to paragraph 1.2. or 1.5. is contacted at 4°C for 24 h. with a solution of 3-chloroperbenzoic acid (0.06M) in diethyl ether (abs.). The surface is subsequently washed with diethyl ether, ethanol and water (4°C).

2.2. Conversion of epoxides into diols:

The surface having epoxide functions prepared under 2.1. is treated with an aqueous solution of pH = 2.5 at 80°C for 1 h. and subsequently washed with H_2O .

2.3. Conversion of olefins into carboxylic acids:

The TiO_2 surfaces treated according to paragraph 1.2. or 1.5. are brought into contact with an aqueous solution of potassium permanganate (0.1M) and NaIO_4 (0.1M) for 20 min. Subsequently, the surface is washed with 0.1M aqueous NaHSO_3 , with ethanol and water.

2.4. Conversion of halides into azides:

A TiO_2 surface treated according to paragraph 1.7. is brought into contact with a solution of NaN_3 (6 mg/ml) in DMF (abs.) for 15 h. and subsequently washed with DMF and water.

2.5. Conversion of azides into amines:

The TiO_2 surface having N_3 groups prepared according to 2.4. is brought into contact with a solution of SnCl_2 in absolute methanol for 4 h. The surface is subsequently washed with methanol and water.

2.6. Activation of carboxylic acids with ethyl chloroformate and N-hydroxysuccinimide:

The surface having COOH groups prepared according to paragraph 2.3. is brought into contact with a 2.5% solution (v/v) of ethyl chloroformate in CH_2Cl_2 /pyridine (100/2.5) for 1 h. Subsequently, the surface is contacted with a solution of N-hydroxysuccinimide (0.5M) in pyridine. There are thus obtained N-hydroxysuccinimide-activated carboxylic acid functions to which molecules having amino groups can be added directly.

3. Modification of organic monolayers prepared according to the procedure described under 2. with low-molecular, hydrophilic compounds.

3.1. Addition of fradiomycin to a TiO_2 surface provided with an organic monolayer:

The surface having activated carboxylic acid functions prepared according to paragraph 2.6. is brought into contact with a solution of fradiomycin (20 mM in PBS; pH = 7.2) for 1 h. It is subsequently washed with H_2O .

3.2. In situ modification of immobilized fradiomycin:

a) Introduction of carboxylic acid functions: The amino groups of the fradiomycin immobilized on the surface according to paragraph 2.1. are quantitatively reacted by contact with a solution (1% w/w) of succinic anhydride in pyridine. There is thus produced a hydrophilic carrier layer which has a high density of available acid functions.

b) Introduction of activated disulphide bonds: 3-(2-pyridinyl)dithiopropionate can be coupled to the amino groups of the fradiomycin immobilized on the surface according to paragraph 3.1 by contact with an ethanolic solution (2 mM) of N-succinimidyl 3-(2-pyridinyl)dithiopropionate. The dithiopyridinyl group can be used for the planned immobilization of molecules (e.g. Fab' fragments of IgG molecules) on the carrier layer via free thio functions. The unreacted amino groups in this procedure can

be used according to the procedure described under a) in order to immobilize carboxylic acid functions on the surface.

c) Introduction of photoactivatable phenylazido groups: 6-(4'-azido-2'-nitrophenylamino)hexanoate can be immobilized on the amino groups of the fradiomycin immobilized on the surface according to paragraph 3.1. by contact with an aqueous (10% DMSO) solution (2 mM) of N-succinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate. Other reactive amino functions can be used according to the procedure described under a) in order simultaneously to modify the carrier layer with carboxylic acid groups.

4. Construction of a porous carrier layer on the organic monolayers for the production of three dimensional recognizing elements:

4.1. Methylcarboxylation of dextran 500000:

75 ml of dry DMSO are added to 7.5 g of NaH. The concentration of thus-obtained DMSO anions is determined by titration. 0.2 equivalent (based on glucose sub-units) of dextran 500000 is dissolved in 150 ml of dry DMSO and the solution is mixed with the DMSO anions. The mixture is stirred at room temperature for 4 h. and added to a two-fold excess (based on glucose sub-units) of bromoacetic acid. The solution is stirred for 12 h. Subsequently, the dextran is precipitated with acetone, filtered off, dissolved in 20 ml of water and dialyzed against water for 24 h. After lyophilization, the amount of methylcarboxylated glucose sub-units is determined by titration (about 1 carboxyl group/5 glucose sub-units).

4.2. Immobilization of methylcarboxylated dextran 500000 on organic monolayers:

The immobilization of methylcarboxylated dextran starts from organic monolayers which are constructed according to paragraph 1.7 on TiO₂ surfaces and which have been modified

according to paragraphs 2.4. and 2.5. The amino groups of these organic monolayers are reacted with epichlorohydrin solution (1 ml of epichlorohydrin in 10 ml of NaOH (0.4M)/10 ml of diglyme). After washing with ethanol and water, it is treated with a solution of methylcarboxylated dextran (0.3 g of dextran in aqueous NaOH solution (0.01M NaOH)) for 24 h. The surface is subsequently washed well with water at 50°C.

5. Preparation of recognizing elements of type A1, A2, B1 and B2 on the basis of the carrier layers referred to in parts 1-4.

5.1. Preparation of recognizing elements of type A1 having immobilized IFN α (interferon α) as the receptor molecule:

For the immobilization of IFN α , a carrier layer is prepared which has been modified with succinic anhydride (coating of TiO₂ according to 1.1., addition of fradiomycin according to 3.1., modification of the fradiomycin according to 3.2.a.). This surface is treated for 5 min. with an aqueous solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (20mg/ml) and N-hydroxysuccinimide (3 mg/ml). After washing with acetate buffer (0.01M; pH = 5.5), it is incubated with a solution of interferon (0.9 μ g/ml) for 20 min. The surface concentration of IFN α achieved using this procedure is 0.36 ng/mm² after washing with acetate buffer and 0.01M HCl.

5.2. Preparation of a recognizing element on TiO₂ according to type A2 with GpIIb-IIIa (glycoprotein IIb-IIIa) as the receptor molecule:

The immobilization of the GpIIb-IIIa starts from a TiO₂ layer which is modified with a diol-containing surface (preparation according to paragraph 1.5., 2.1. and 2.2.). This surface is treated with a 0.5% solution of 1H,1H,2H,2H-perfluorooctyldimethylchlorosilane in CCl₄. The thus-obtained strongly hydrophobic surface is brought into contact with an aqueous solution of GpIIb-IIIa (0.5 μ g/ml) (0.1M Tris; pH = 7.2) for

20 min. The surface concentration of GpIIb-IIIa achieved using this procedure is 1.5 ng/mm² after washing with buffer solution.

5.3. Preparation of a recognizing element of TiO₂ according to type B1 having TNF α (tumor necrosis factor α) as the receptor molecule:

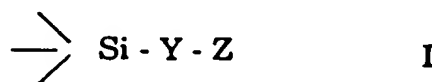
A TiO₂ surface modified with methylcarboxylated dextran according to paragraph 1.5., 4.2. is used for the immobilization of TNF α . This surface is treated for 5 min. with an aqueous solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (20mg/ml) and N-hydroxysuccinimide (3 mg/ml). After washing with acetate buffer (0.01M, pH = 5.5), it is incubated with a solution of TNF α in acetate buffer 1 μ g/ml for 10 min. According to this procedure and after washing with acetate buffer, PBS buffer (0.1N; pH = 7.2) and ethanolamine (1M, pH = 8.5), about 2 ng/mm² of TNF α are covalently immobilized.

5.4. Preparation of a recognizing element on TiO₂ according to type B2 with antibodies as receptor molecules:

The directed immobilization of the antibodies is effected on a dextran carrier layer which has been prepared according to paragraph 1.5., 4.2. For the directed immobilization, free aldehyde functions are produced on the carbohydrate residues of the antibodies by oxidation according to known procedures. The dextran layer on the TiO₂ wave guide is treated for 5 min. with an aqueous solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (20mg/ml) and N-hydroxysuccinimide (3 mg/ml). After washing with water, the activated carboxylic acid functions on the surface are converted into hydrazides by contact with an aqueous solution of hydrazine monohydrochloride (1 mM). This surface is brought into contact with a solution of the oxidatively-treated antibodies (1 μ g/ml in acetate buffer (0.01M, pH = 5.5)) for 20 min. The surface is washed with PBS (0.1M; pH = 7.2) and an aqueous solution of ethanolamine (1M; pH = 8.5). This procedure leads to a directed immobilization of about 5 ng/mm² of antibodies on the dextran carrier layer.

Claims

1. An optical biosensor comprising a dielectric wave guide and an organic carrier layer to which receptor molecules are bonded, wherein the organic carrier layer forms a monomolecular layer and consists of molecules of general formula I



and wherein the molecules of the carrier layer are bonded directly to a TiO_2 wave guide via the Si atom or, if desired, are bonded indirectly to the TiO_2 wave guide via an intermediate layer.

2. An optical biosensor according to claim 1, wherein group Y is a spacer group and groups Z are hydroxyl, carboxyl, amine or methyl, alkyl or fluoroalkyl groups.

3. An optical biosensor according to either of claims 1 and 2, wherein groups Z are derivatives of hydrophilic, short-chain molecules such as oligovinyl alcohols, oligoacrylic acids, oligoacrylic acid derivatives, oligoethylene glycols or mono- or oligo-saccharides having 1-7 sugar units or carboxyglycosides or aminoglycosides having 1-5 sugar units.

4. An optical biosensor according to any one of claims 1-3, wherein the aminoglycosides are derivatives of compounds such as fradiomycin, kanamycin, streptomycin, xylostasin, butirosin or chitosan.

5. An optical biosensor according to any one of claims 1-4, wherein groups Z are hydrogel-forming groups.

6. An optical biosensor according to any one of claims 1-5, wherein the hydrogel-forming groups are derivatives of polysaccharides such as dextran, agarose, alginic acid, starch,

cellulose and derivatives of such polysaccharides or hydrophilic polymers such as polyvinyl alcohols, polyacrylic acids, polyethylene glycols and their derivatives.

7. An optical biosensor according to any one of claims 1-6, wherein the intermediate layer is a thin layer ($d < 20$ nm) of SiO_2 or Al_2O_3 .

8. A method for the production of an optical biosensor according to any one of claims 1-7, which method comprises using compounds of general formula II



II

wherein X is hydrogen, fluorine or a chemically reactive group, $\text{R}^1\text{R}^2\text{R}^3$ is alkyl, alkoxy or halogen and Y is a spacer group,

for the production of the ordered monomolecular layer and applying these compounds to the TiO_2 wave guide either from the gas phase or from solution and, if desired, subsequently modifying group X by oxidation, reduction, substitution or addition such that group Z results and the receptor molecule can be coupled to group Z.

9. A method for the production of an optical biosensor according to claim 8, wherein group Z is modified by addition, substitution, oxidation or reduction such that it carries groups, molecules or molecular associations which permit the concentration of receptor molecules in a directed or non-directed manner.

10. A method for the production of an optical biosensor according to claim 8 or 9, wherein group Z is modified by addition, substitution, oxidation or reduction such that it subsequently carries photoreactive groups.

11. The use of an optical biosensor according to any one of claims 1-10 for the determination of the concentrations of an

analyte molecule in solution or for the quantification of an interaction between analyte and receptor molecule based on thermodynamic and kinetic data.